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Perspective

Toward the massive genome of *Proteus anguinus*—illuminating longevity, regeneration, convergent evolution, and metabolic disorders

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Deciphering the genetic code of organisms with unusual phenotypes can help answer fundamental biological questions and provide insight into mechanisms relevant to human biomedical research. The cave salamander *Proteus anguinus* (Urodela: Proteidae), also known as the olm, is an example of a species with unique morphological and physiological adaptations to its subterranean environment, including regenerative abilities, resistance to prolonged starvation, and a life span of more than 100 years. However, the structure and sequence of the olm genome is still largely unknown owing to its enormous size, estimated at nearly 50 gigabases. An international Proteus Genome Research Consortium has been formed to decipher the olm genome. This perspective provides the scientific and biomedical rationale for exploring the olm genome and outlines potential outcomes, challenges, and methodological approaches required to analyze and annotate the genome of this unique amphibian.

Keywords: olm; large genome sequencing; biomedical application; subterranean environment; conservation genomics

The subterranean environment poses fundamental challenges to animals that inhabit it. While adaptations enabling the survival in harsh underground environments may appear unusual at first glance, some traits that have evolved in subterranean animals are highly relevant for addressing evolutionary and developmental questions, as well as for biomedical research, including understanding the mechanisms behind circadian rhythms, longevity, starvation, and regeneration.[1]

The cave salamander, *Proteus anguinus* (Urodela: Proteidae), also known as the olm (or simply proteus), is the first described cave organism and the largest known obligate cave tetrapod in the world (Fig. 1). This unusual species is endemic to the subterranean waters of the Dinaric Karst of Southeastern Europe and highly adapted to the underground environment. Besides morphological traits, such as depigmented skin, degenerated eyes, and elongated body parts,[2] olms display physiological adaptations to oxic and oligotrophic conditions. These include high tolerance to anoxia,[3] slow metabolism, and resistance to starvation for up to 8 years.[2] In the eternal darkness of subterranean waters, olms have evolved a wide array of nonvisual sensory systems. In addition to enhanced chemo- and mechanoreceptive capabilities commonly found in cave vertebrates, these sensory systems include electroreception, photosensitive skin pigments, magnetoreception, and auditory abilities in a range of

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Figure 1. **The olm (*Proteus anguinus*). Some of the emblematic features of cave animals (lack of eyes and pigmentation, elongated head and body) are visible.**

frequencies far above that of most aquatic vertebrates.[4] With an estimated life span of more than 100 years, olms are among the longest living vertebrates[5] and, like other urodeles, are able to fully regenerate damaged limbs and organs.[6] Olms are neotenic and retain their larval characteristics—such as external gills, gill slits, and skin morphology—throughout their life.[2]

Research into the genetics and biology of the olm's adaptations holds promise for better understanding and possibly combating human ailments such as aging, degeneration (including loss of body parts), and obesity. As a first step, sequencing and assembly of the genome is critical to achieve this goal. The olm qualifies as one of the "fantastic beasts," organisms with surprising adaptations, whose genomes have recently received much attention in ecological genomics and as sources of new biomedically relevant information.[7,8] Much progress has been made on understanding the genetics of starvation resistance and obesity using cavefish.[7,9,10] However, as tetrapods, olms are phylogenetically closer to humans than other model organisms such as cavefish and, therefore, a potentially useful comparative model. Since there are no obligate cave mammals—not even amniotes—the olm is arguably the most faithful mirror of genomic cues for human medical conditions that have their counterparts in cave-related traits.[7,11]

Adaptation and evolution of subterranean animals

Understanding the genetic mechanisms underlying the adaptation of organisms to new environments is one of the fundamental goals of evolutionary biology. In deciphering these mechanisms, organisms under strong selection pressure from extreme environments, including subterranean habitats, have proven particularly useful.[12]

In the evolutionary transition from a surface-dwelling to a subterranean lifestyle, populations are faced with perpetual darkness, nutrient scarcity, constant temperature, high humidity, and impoverished species communities. Established cave species often evolve a set of characteristic phenotypic traits known as troglomorphisms. Several of these traits, for example, depigmentation, eye loss, and elongation of body parts, show a high degree of convergence among various groups of subterranean animals. One of the best-known examples of such convergent evolution is the olm. It has been on the stage of evolutionary biology since Darwin's mention of it in *On the Origin of Species*.

The olm comprises several genetically distinct lineages,[13] with varying degrees of troglomorphism. In contrast to the blind Mexican cavefish, *Astyanax mexicanus*, a classic cave vertebrate model organism that has only recently colonized caves,[14] the olm might have been dwelling in caves for over 10 million years. Yet, one lineage has retained the ancestral phenotype with dark pigmented skin and structurally intact eyes. This makes olms a nearly ideal study system for understanding the genetic mechanisms driving the evolution of cave-associated phenotypes and the process of convergent evolution. For example, dark pigmentation and the core development of the eye are regulated by deeply conserved pathways among animals.[15,16] Researchers are currently debating the extent to which the loss of pigment in the eye is due to positive directional selection, antagonistic pleiotropy, or the accumulation of selectively neutral but phenotypically destructive mutations.[17,18] Here, the olm presents a phylogenetically older alternative to *Astyanax*.

Sequencing and annotation of the olm genome will allow identification of genes under positive and relaxed selection,[19] compared with other fully sequenced vertebrates. These genes will be functionally characterized, grouped, and linked to a "cave phenotype," with particular focus on traits with biomedical relevance.

Resisting senescence—the evolution of longevity

With a maximum predicted life span of over 100 years, olms outlive other amphibians by decades and have one of the longest life spans known in

vertebrates considering their low body mass that rarely exceeds 50 grams.[5] Capable of reproducing at over 80 years of age,[20] olms show no age-related decline in fecundity and exhibit negligible signs of senescence. Although the longevity of the olm can be attributed in part to its low basal metabolic rate,[2] the exact mechanisms behind its long life span remain unknown.

These features make the olm a suitable animal for longevity studies. In addition to the potential identification of positively selected variants or combinations of longevity-associated genes known from other vertebrates,[21] we may expect new candidate genes and pathways associated with life span in the extreme subterranean environment. Most biomedical research related to the genetics of aging and longevity has traditionally been based on closely related mammals, or on evolutionary very distant invertebrates, such as *Caenorhabditis elegans*.[22,23] Olms represent an independent vertebrate model that evolved long life span. The stark contrast of a long-living exception in a group of generally short-lived amphibians with already sequenced genomes[24,25] may allow the prioritization of genes related to longevity, which makes the olm attractive for a comparative genomic approach.

Evolution of developmental and physiological adaptations

Some of the typical phenotypic traits of cave animals (e.g., loss of vision, pigmentation, and circadian rhythm) closely resemble pathological conditions and developmental disorders of other organisms, including humans. Because the same genes are often involved in the underlying mechanisms in humans as well as in other cave vertebrates, such as cavefish, the genomes of these organisms may hold the key for better understanding the genetic basis of sleep and behavioral disorders, albinism, and abnormalities in eye development.[7,10] Cave vertebrates are capable of prolonged starvation but also of feasting when food is abundant, without developing obesity or insulin resistance. Their genomes may deliver new insights into metabolic pathways regulating efficient absorption and storage of nutrients, enabling prolonged starvation and survival at low metabolic rates. This holds the promise of better understanding the genetic mechanisms behind metabolic and nutritional disorders in humans.[9] Along with efficient handling of starvation, the genome of the olm may also help to understand the immune response mechanisms against pathogens and parasites during periods of intense energy conservation. Their functioning is largely unknown, but new genomic insights from the olm may improve our understanding of the hierarchy of defense mechanisms in vertebrates.

Like most urodeles, olms are capable of complete regeneration of damaged tissues and organs. A comparison of the olm genome to that of other salamanders, particularly *Ambystoma mexicanum*, a well-known model organism in regenerative biology, is expected to shed light on the common genetic grounds of regeneration.[26] This will expand the possibilities of understanding and applying this unusual trait in biomedicine.

Species conservation

The apex predator of the Dinaric underground waters, the olm has a significant ecological role. It is an indicator of the health of the karstic groundwater ecosystem and a pointer to the absence of pollution of underground water resources used for human consumption. The range of the species is confined to an area reaching from the far northeast of Italy via Slovenia, Croatia, and Bosnia and Herzegovina to Montenegro, with the largest number of known localities found in Slovenia. Due to its endemics, narrow ecological niche, fragmented distribution, and threats to its habitat, the species is listed as vulnerable in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species[27] and protected by national legislations.

The inaccessibility of the subterranean habitat has largely precluded accurate estimates of population size, structure, and distribution. This poses major obstacles to developing effective conservation measures. Novel conservation genomic approaches can help to overcome some of these challenges.[28] The expected benefit of genomic data from multiple individuals and populations includes a clear delimitation of evolutionarily significant units, long-term population size changes, as well as a better understanding of issues related to olm health, especially susceptibility to disease and other environmental stressors.

Genomic data can further support an applied approach to conservation by *ex situ* breeding programs. The progress of such programs is currently

hampered by the difficulty of reliably determining the sex of animals by external or nondestructive internal means. Recent cytogenetic research revealed that the absence of sexual dimorphism in olm might be related to a translocation of sex chromosomes to autosomes.[29] We expect that genomic analysis will reveal sex-related genes, depending on the reliability and noninvasiveness of the sexing assay. Furthermore, sex determination in amphibians is diverse: numerous independent transitions have been documented throughout the phylogeny.[30] Therefore, it is essential to examine the genetic basis of sex determination in different amphibian groups.

Large genome size and repetitiveness

Although the olm genome is of great interest to the scientific community, the information encoded within it remains unknown due to its large size. With an estimated size of 48.84–51.50 Gbp,[31] the olm genome is comparable to the largest sequenced animal genomes so far, that is, lungfishes[32,33] and axolotl.[25] Also, due to the relatively large evolutionary distance between these species, the olm genome is expected to provide new insights into the evolution of large genomes and advance our understanding of the role of noncoding DNA prevalent in genomes of eukaryotes, including humans.

The impact of repetitive regions on the functionality of the organism and its genome is currently not well explored, in part because of the lack of assembled genomes with large amounts of repetitive DNA. Long repetitive DNA is increasingly recognized as an essential part of the genome, contributing to functionality,[34] structural integrity, and genome architecture,[35] and has been speculated to even be involved in the evolution of novelty.[36] While the assembly of large repetitive regions, such as in lungfishes[32,33] or salamanders,[25] remains a serious challenge,[37] this obstacle can now be overcome with long-range third-generation sequencing technologies. Quantifying and characterizing the amount and type of repetitive DNA in the olm genome is one of the aims of our project. Identification of differences and similarities between the olm genome and other sequenced animal genomes with large amounts of repetitive DNA will provide new insights into the evolution of repetitive DNA and its role in animals.

The Proteus Genome Research Consortium

To decode the olm genome and identify the genetic variation determining the outstanding phenotype of the species, a scientific consortium was formed, consisting of experts from the University of Ljubljana (Slovenia), Aarhus University (Denmark), Lars Bolund Institute for Regenerative Medicine (China), Jožef Stefan Institute (Slovenia), and representing the biotechnological capabilities of BGI Research (China) and MGI Tech (China and Latvia). The goal of the consortium is to sequence and assemble the genome of the olm *P. anguinus* (http://proteusgenome.com). To circumvent the technical challenges presented by one of the largest known vertebrate genomes,[37,38] the consortium will pursue an integrative approach, combining various technologies, with a focus on long-range sequencing methods (Fig. 2). First, high molecular weight DNA from several tissues will be sequenced with single-molecule long-read sequencing, including the real-time sequencing of Pacific Biosciences to generate long-read genome scaffolds.[39] Next, DNA nanoball-based high throughput short-read sequencing and single-tube long-fragment read sequencing (stLFR) will be used. Short reads will be mapped to the scaffolds and used for sequence correction.[40] Following that, Hi-C scaffolding[41] will be performed to generate a chromosome-scale assembly, with custom-adapted bioinformatic algorithms[33] to overcome the scaffolding obstacles presented by the large amount of repetitive regions.[32,33] Finally, total RNA from major tissues/organs (including brain, heart, lungs, liver, kidney, spleen, stomach, small intestine, duodenum, colon, blood, muscle, testicles, and skin) will be sequenced by a combination of short- and long-read sequencing technologies and used to guide the structural gene annotations.

The olm is listed as vulnerable by the IUCN and highly protected by EU and national legislations. Therefore, obtaining the tissue samples appropriate for high-quality molecular DNA and RNA extractions was performed with utmost diligence to minimize the impact of the sampling on the wild olm population in accordance with all the relevant laws and provisions on animal protection and welfare. Individuals from the Postojna-Planina Cave System in Slovenia were chosen. While historically this

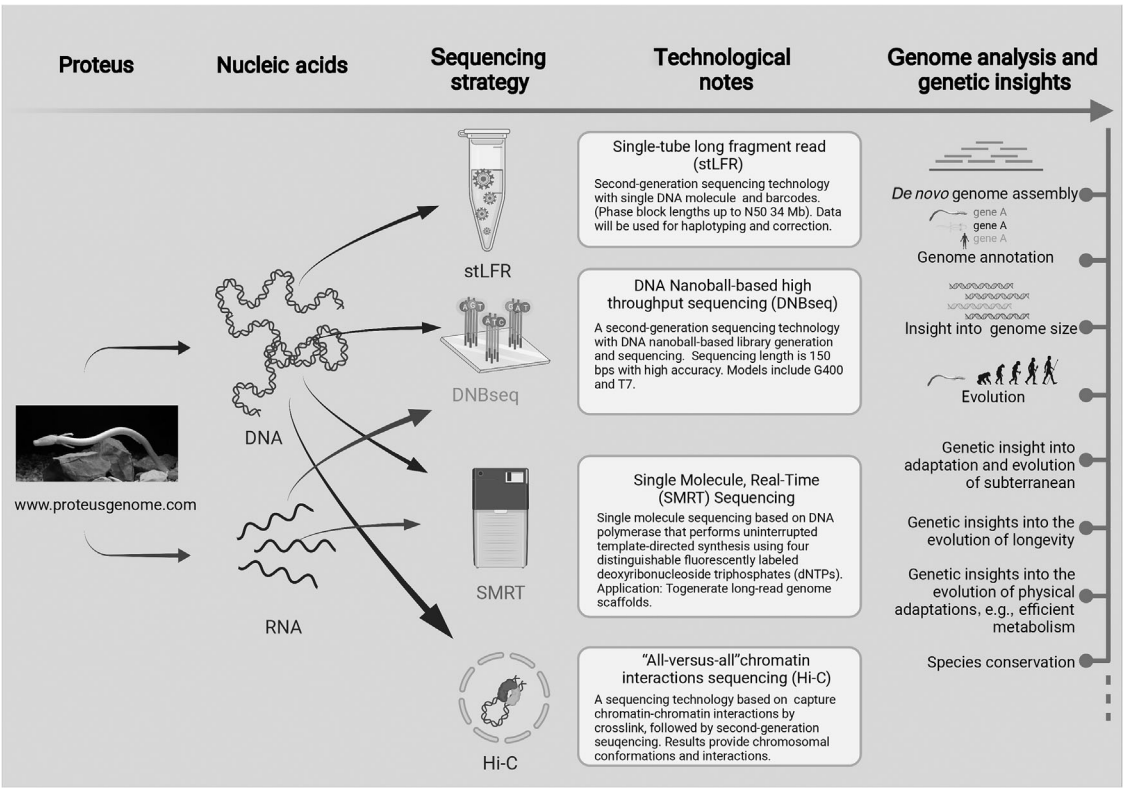

Figure 2. **Schematic illustration of the olm genome sequencing plan. Both genomic DNA and RNA are isolated from olm tissue, followed by four sequencing strategies: stLFR, DNBseq, SMRT, and Hi-C. The proteus genome will provide genetic insights into the evolution and adaptation to the subterranean environment, longevity, metabolism, regeneration, and aid species conservation (created with BioRender.com).**

location has suffered from overcollection of individuals, after stricter protection measures were undertaken in the late 20th century, it currently contains a stable and healthy population.[42] This cave is also the type locality of the species, and therefore any potential future changes in taxonomy will not affect the species name of the individual designated for genome sequencing.

Conclusion

The Proteus Genome Research Consortium (http://proteusgenome.com) has been established to tackle the challenge of sequencing the olm, *P. anguinus*. Pilot data from the crude genome sequence of estimated size over 40 Gbp and preliminary transcriptome sequence data from several tissues have since been obtained. Sequencing is now entering the next phase with single-molecule real-time sequencing as well as short-read sequencing and transcriptome sequencing. We expect that even before the chromosome-level assembly of the genome is achieved, these data can be analyzed to provide novel insights into the surprising biological, developmental, and evolutionary features of this cave-dwelling salamander. We also expect that these insights will have important implications for improving human health and will advance medical progress in fields such as aging, regeneration, and metabolic disorders. Despite the challenges associated with the size of the olm genome, recent success in sequencing, assembly, and annotation of other large animal genomes[25,32,33] suggests that the time is now ripe to decipher the genetics of *P. anguinus*, one of the planet's most unusual creatures.

Author contributions

R.K., B.D., N.G.-C., L.B., and Y.L. contributed to the study concept and design. C.G., H.R., G.F., and H.J. retrieved and analyzed the data. R.K., B.D., Y.L., and H.R. drafted the paper, and all authors provided

critical revisions and approved the final version of the manuscript for submission.

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Competing interests

The authors declare no competing interests.

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